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Development of transgenic pigeonpea (*Cajanus cajan*. L Millsp) overexpressing citrate synthase gene for high phosphorus uptake

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Plants have developed several adaptive strategies to enhance the availability and uptake of phosphorus (P) from the soil under conditions of P deficiency. Exudation of organic acids like citrate is one of the important strategies. In this study, we developed transgenic pigeonpea (*Cajanus cajan*) over-expressing *Dacus carota* citrate synthase (*DcCs*) gene to increase the synthesis and exudation of citrate. Transgenic plants were generated through agro bacterium mediated *in-planta* transformation technique. Integration and expression of the transgene was confirmed by genomic Southern and RT-PCR analysis. We observed that the transgenic lines had more tissue P and chlorophyll content, and also citrate synthase content higher in the roots. Further, transgenic lines had more vigorous root system both under P sufficient and deficient conditions with more lateral roots and root hairs under P deficient conditions. We conclude that the transgenic pigeonpea plants have the capacity to acquire more P under P deficient conditions.

Keywords: Crop productivity, Soil nutrients

For plants, low availability of Phosphorus (P) due to its low mobility and higher fixation in the soil are major constraints limiting its uptake and utilization, thereby reduced productivity. The concentration of inorganic P in the soil solution is often as low as 2-10 µM[1]. Consequently, the supply of phosphorus to the root surface by diffusion is also very slow[2]. Hence, phosphorus, a major nutrient involved in plant growth processes, is the most unavailable and inaccessible macro nutrient in the soil[3].

Because of its unique interaction with other elements, up to 80% of applied P is not available to the plants but fixed to the soil. Depending on the soil reactions, P may either be bound to Ca and Mg in calcareous soils or to Fe and Al in soils with acidic reaction, leading to huge buildup of non available P in the soils. This has resulted in application of higher amounts of phosphatic fertilizers every season which may either get fixed again or lost through runoff finding its way to the fresh water bodies leading to eutrophication.

As an adaptive response to low phosphorus, plants have evolved several strategies such as formation of cluster roots[4], increased number of lateral roots and root hairs[5,6], modification of rhizosphere by exudation of organic acids[6-8] and increased synthesis of enzymes like acid phosphatase[9,10], to acquire more phosphorus under deficiency.

In most of the plants, citric acid is the major organic acid released under P deficient conditions[11]. Increased synthesis of citrate synthase results in increased production of citrate[12]. The released citric acid is known to solubilize the inorganically bound P in the rhizosphere and enhance its uptake[12].

Pigeonpea, an important food legume of the arid and semi arid tropics, generally grown as a rain fed crop, has fairly higher requirement of P for its normal growth and productivity. Considering the scarce availability of P in the soils and also the fact that the natural resources of P are dwindling fast and would be exhausted in the next 35-40 years, we attempted to develop transgenic pigeonpea plants over-expressing citrate synthase gene cloned from *Dacus carota* (*DcCs*) under a constitutive and a root specific promoter. We report here, the performance of transgenic plants overexpressing *DcCs* gene under phosphorus deficient conditions.

Materials and Methods

Plant material

Pigeonpea (*Cajanus cajan*), variety TTB-7 was used to generate transgenic plants. TTB-7 is one of

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the ruling varieties of Karnataka being grown by farmers from the last 20-25 years. Seeds were soaked in 0.1 % (W/V) carbendezim overnight followed by surface sterilization with 0.1 % (W/V) $HgCl_2$, washed thoroughly with distilled water and placed for germination in Petri plates with double layer of blot paper discs to hold sufficient moisture at 30°C. Two day old seedlings were used as explants for *Agrobacterium* mediated *in-planta* transformation.

Gene constructs for transformation

The cloned *DcCs* gene was obtained from Dr. Koyama, Faculty of Agriculture, and Gifu University, Japan. PCR based cloning approach was followed to $AtPT_2$ root specific promoter from *Arobidopsis thaliana*. The primers were designed based on Arabidopsis genome database (TAIR) and NCBI database. The pLPTV plasmid containing $AtPT_2$ was procured from USA. The pLPTV:$AtPT_2$ plasmid was used as template to amplify the promoter under standardized PCR conditions using *pfu* DNA polymerase. The primers used for PCR amplification are shown in table 1. The amplified products were fractionated on 0.8% agarose gell and eluted using GenElute™ gel extraction kit (Sigma). The eluted fragments were cloned with InsT/Aclone PCR product cloning kit (Fermentas life sciences), confirmed by restriction profile, sequence and blast analysis (*http*://www.ncbi.nlm.nih.gov/Blast)

Two constructs were developed, one under constitutive (*CaMV 35S*) and another under a root specific ($AtPT_2$) promoter, with *nptII* as a selectable marker, separately in two destination vectors, namely pK7WG2.0:$AtPT_2$ and pK7WG2.0, through gateway cloning approach. The *Agrobacterium* strain used was GV3101. Schematic representation of both the constructs is given in Fig. 1

Plant transformation and selection of transformants

Transformation was accomplished using tissue-culture independent *Agrobacterium* mediated *in-planta* transformation technique[13]. The seedlings with just emerging plumule were infected by wounding the meristematic tissue with fine sterile needle and subsequently incubating in *Agrobacterium* culture carrying the constructs of *DcCs* for 1 h. Following infection, the seedlings were washed with sterile water and later transferred to wide mouth capped glass jars of 300 mL capacity, containing sterile soil rite (Vermiculite equivalent; Keltech Energies Ltd., Bangalore, India) sufficiently moistened with water for further growth. The infected seedlings were incubated in a growth chamber maintained at 28±1°C under a 12 h photoperiod with fluorescent light (FL40S.W, Mitsubishi, Tokyo) of 35 µ moles m^{-2} s^{-1} intensity. After 8-10 days, the seedlings were transferred to earthen pots with a capacity to hold about 15 kg autoclaved red loamy soil and a dose of fertilizer @ 25:50:25 N:P:K (EID Parry Pvt. Ltd., Chennai, India) kg ha^{-1} was applied. All the plants (T_0) were maintained in a transgenic containment facility with optimum temperature of 32°C and a relative humidity of 85%. Subsequently, seeds from these plants were obtained, screened and advanced to T_1, T_2 and T_3 populations under similar conditions. All the transgenic plants from T_1 to T_3 generation were grown in normal autoclaved soil. For further physiological and biochemical studies, 5 lines of $AtPT_2$::*DcCs* construct and 4 lines of *CaMV*::*DcCs* construct which were southern positive were used, and these lines were grown in perforated thermocol cups holding equal amount of soil rite. Once the roots come out of the perforations, the cups were mounted on to thermocol sheets with holes to hold these cups kept on plastic trays containing half strength Hoagland's nutrient solution.

From T_1 generation, 53 PCR positive and kanamycin positive lines of $AtPT_2$::*DcCs* construct and 35 lines of *CaMv*::*DcCS* construct were forwarded to T_2 generation. From T_2, 12 lines of $AtPT_2$::*DcCs* construct and 6 lines of *CaMv*::*DcCS* construct were selected and carried forward to T_3 generation and based on physiological and molecular data, Five line.(T_3) of $AtPT_2$::*DcCs* construct and 4 lines (T_3) of *CaMv*::*DcCS* construct were used for the genomic Southern using gene specific (*DcCs*) and marker specific (*nptII*) probes.

Table 1—Primers designed to clone $AtPT_2$ root specific promoter from *Arobidopsis thaliana*

Primer	Sequence	Product size
$AtPT_2$-FP	CCCATTCGTGAATGAAAACTACGGTG	2.3 kb
$AtPT_2$-RP	CTTCTTCTCCTCTGCAATTTTTCATC	

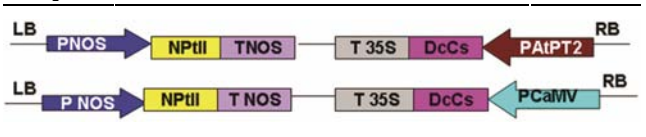

Fig. 1—Schematic representation of the constructs developed: *CaMV35 S* promoter was released from pK7WG2.0 using HindIII and SpeI and root specific promoter *AtPT2* was cloned in place of 35S of pK7WG2.0. Theses constructs were cloned in two different destination vectors (pK7WG2.0:*AtPT2* and pK7WG2.0) for plant transformation.

Molecular analysis of the transformants

PCR analysis

PCR analysis was done as per[14]. Genomic DNA was isolated from both wild type and transgenic plants. The transformants were initially tested for integration of the transgene by PCR analysis, using a set of gene specific primers designed for *DcCs* gene and root specific promoter ($AtPT_2$) (Table 2). The DNA fragments were amplified from genomic DNA in a 20 µL reaction volume containing 0.1 µg of DNA template, 2.0 µL of PCR buffer (10X), 2.0 µL of dNTPs (2 mM), 1 U Taq DNA polymerase (Engene Pvt Ltd., Bangalore) and sterile double distilled water (to make up the volume to 20 uL). PCR was performed by keeping plasmid DNA as a positive control and water blank as a negative control. Standard PCR program was carried out by initial denaturation at 94°C for 4 min followed by another denaturation at 94°C for 1 min and then Primer annealing at 58°C for 1 min and DNA synthesis at 72°C for 1 min followed by denaturation again at 94°C for 1 min (30 cycles) with a final extension at 72°C for 10 min and held at 4°C.

The PCR amplified samples were analyzed by running in 0.8% agarose gel electrophoresis. The presence of the expected products was determined with the help of DNA ladders (Gene ruler DNA ladders, MBI-Fermentas). The authenticity of obtained expected bands (427 bp with *DcCs* and 740 bp with $AtPT_2$ primers) were checked by sequencing the amplified product.

Southern analysis

Southern blot analysis was performed to check the stability of the transgene. Genomic DNA was isolated as per[15] from the selected promising lines grown in the green house facility and southern analysis was carried out as per the method of Southern[16]. About 20 µg of genomic DNA was digested with Hind III and EcoR V. The reaction was carried in final volume of 30 µL with DNA, buffer (1X) and restriction enzymes (5U mg^{-1}). The reaction mixture was incubated at 37°C overnight. The reaction was stopped by incubating at 65°C for 15 min. The digested samples were run on 0.8% agarose gel along with gene ruler. The gel was depurinated by incubating for 15 min with 0.15N HCl, then 45 min with denaturing buffer (1.5 M NaCl:0.5 M NaOH) and finally incubated for 45 min with neutralization buffer (1 M Tris-HCl: 1.5 M NaCl, pH 8.0) on a shaker. The digested DNA from agarose gels was transformed on to 0.45 µM nylon membrane as described by Southern[16]. *NptII* fragment (750 bp) probe was prepared with ^{32}P dCTP (BARAC, Trombay, Mumbai, India), using BRIT random primer kit for DNA labeling and purified the labeled probe using Sigma Spin™ post reaction clean up columns (Sigma-Aldrich, USA). The nylon membrane was pre-hybridized at 65°C with Maxum-Gilbert buffer (0.5 M Sodium phosphate buffer, pH 7.2, 10 mM EDTA, 7% SDS) for 6 h in a rotating hybridization oven. The pre-hybridization solution was replaced with fresh pre-hybridization solution, and the heat denatured probe (immediately chilled on ice) was added and incubated overnight at 60°C. The membrane was subsequently washed with 2X SSC and 1X SSC along with 0.1% SDS for 15 min at 28°C The membrane was then dried and wrapped in saran wrap and exposed to phosphor image plate and the image was read using Phosphor Imager (FLA 5100, Fuji Film Co. Tokyo, Japan).

RT-PCR analysis

Reverse transcriptase polymerase chain reaction (RT-PCR) was done as per Destefano *et al.*[17]. Total RNA was isolated from the selected promising lines by following the phenol-chloroform method[14]. About 5 µg of total RNA was reverse transcribed using MMLV (Molony Murine Leukaemia Virus reverse transcriptase enzyme. Quantitative RT-PCR was done to study the expression levels of *DcCs* gene using gene specific primers. First strand of cDNA was used

Table 2—Specific primers, their product length and annealing temperature

ID	Sequence	Primer length	Product length	Annealing temp.
Sequences of the gene specific primers used in the PCR analysis				
DcCS-FP	5' GGTGGAGAACCATTGCCTGAG 3'	21	427 bp	58°C
DcCS-RP	5' GCTCTTGCATGCTGGGACTATC 3'	23		
Sequences of the primers designed for root specific promoter				
AtPt2-FP	5' CTCCCGTACCCGTTCAAACTACAC 3'	24	740 bp	59°C
AtPt2-RP	5' CTGCCTATGAACAAGGACAG 3'	20		

as a template for PCR reaction, as the RNA can't be amplified directly. RNA was converted to cDNA with the help of reverse transcriptase. Initially, 10 µL reaction mixture [comprising 5 ug of total RNA, 1 µL of Oligo (dt) primer (10 pmol/µL) and RNase free sterile distilled water] was incubated at 65°C for 10 min (to remove any secondary structure) and immediately placed on ice. About 10 µL of another reaction mixture [2 µL of dNTPs (10 mM), 4 µL of M-MLV RT buffer (5X), 50 U of M-MLV and sterile water] was added and briefly spinned. The resulting 20 µL reaction mixture was incubated at 42°C for 90 min to get cDNA.

The primers used were (Forward 5' GCGGGA AGGTTGTTCCTGGATATGG 3'; and Reverse 5' CAG CAGTACCCCACTATGGGCATC 3'. Elf was used an internal control using specific primer; (Forward 5'TCTCAAGCGTGGGTTTGTTGCT 3'; Reverse 5'TTGACAGCAATGTGGGAGGTGT 3'), and the PCR was carried out for the transgene *DcCs*. Reverse transcription was also performed to study the expression pattern of *DcCs* gene. One µL of the cDNA mix was used as template for PCR amplification of a 215 bp fragment

Dot blot analysis

Dot blot analysis was carried out following the method[18]. PVDF membrane was activated using 10% methanol, 250 ng of protein extracted from the sample was spotted on to the membrane and dried. The membrane was then blocked using 3% BSA and incubated with primary antibody, diluted 1:10000 times and kept on a rocker for 2 h, washed 3 times using 0.1% PBST, and later treated with 1:5000 dilution of secondary antibody, incubated on rocker for 2 h followed by 3 washings with 0.1% PBST. Further, 3 mg of DAB dissolved in 5 mL each of citrate buffer and water was added and kept on a rocker for colour development, washed 3 times again using 0.1% PBST.

Expression analysis of citrate synthase

Direct antigen coated enzyme linked immuno-sorbent assay (DAC-ELISA) was carried out for quantification using polyclonal antibodies developed against citrate synthase as per[19]. The ELISA plates were coated using primary antibodies diluted 1:5000 times, using coating buffer and kept overnight at 4°C, washed using 0.1% PBST, followed by incubation with secondary antibodies diluted 1:5000 times, in citrate carbonate buffer for 2 h. After washing with 0.1% PBST, the plates were incubated at 37°C with 3 mg DAB in 5 mL of citrate buffer and 5 mL of water and OD was recorded.

Tissue P content

Leaf samples were collected at the end of treatment period dried in an hot air oven for 48 h at 80°C. Dried and crisp leaf samples were ground to fine powder and the powdered samples were digested using di-acid mixture in a digester (MARS) and diluted samples were analyzed for total P using ICPOES (Thermo fisher).

Physiological analysis of the transformants

Transgenic (T_3) and wild type plants were grown in thermocol cups perforated at the bottom, containing soil rite as the growth medium under normal conditions in quarter strength Hoagland's nutrient solution with sufficient levels of phosphorus and other nutrients for 20 days in two sets. After 20 days when the roots started emerging from the perforations, the cups were fixed into holes made in thick thermocol sheets holding the cups tightly. The thermocol sheets were placed on to plastic trays holding half strength Hoagland's nutrient solution. One set was continued in half strength Hoagland's nutrient solution with P (phosphorus sufficient) and the other with half strength Hoagland's solution without P (phosphorus deficient) for a period of 25 days. After 25 days of treatment, plant samples were collected for quantification of citrate synthase by ELISA and for estimation of tissue phosphorus concentration.

Chlorophyll content

Total chlorophyll content was estimated as per Hiscox & Israelstam[20], by submerging the leaf tissue in 80 % Acetone:DMSO in 1:1 proportion mixture overnight and extracting the chlorophyll from the leaves. Optical density of the solution was recorded at 665 nm and the total chlorophyll was calculated using standard formula.

Root growth pattern

Both transgenic plants (T_3) and non transgenic plants grown in the thermocoule cups perforated at the bottom as mentioned above were used to assess the differences in root growth. Since destruction of sampling was not possible as these plants could not be sacrificed (they had to be grown to obtain their seeds), only a comparative visual assessment of the root length, number of lateral roots and root hairs was done at the end of the experiment.

The data was analysed using factorial RCBD analysis with 5 replications in each experiment.

Results

Molecular analysis of the transformants

PCR analysis with gene specific and npt II primers

PCR analysis of the T_1, T_2 and T_3 transgenic lines, confirmed the integration of *DcCs* gene both under constitutive (*CaMV35S*) and root specific ($AtPT_2$) promoters. The integration was checked by using gene specific primer of 427 bp, root promoter specific primer of 740 bp and marker specific primers of 750 bp. Data of T_3 lines with gene specific and marker specific primers indicated integration of the *DcCs* gene (Fig. 2 A & B), and that of the promoter specific primers (Fig. 2C) confirming the integration of transgene.

Southern analysis

Southern blot analysis of the T_3 generation over-expressing *DcCS* under both root specific and constitutive promoters was done using *nptII* as a probe. The analysis confirmed the integration of transgene and indicated the existence of both single copy and more than one copy number of the gene in the lines tested. However, one of the line numbers 3-1-3 of *CaMV*::*DcCs* construct at lane number 7 indicated a single copy of the gene (Fig. 3A). Expression was very mild in lane number 8 and 9. However, presence of bands in lanes 1-7 indicated integration of the gene.

RT-PCR analysis for transcript accumulation

The RT-PCR analysis was carried out in all the southern positive lines to observe the expression of *DcCs* gene under both root specific and constitutive promoters. Expression of the transgene was apparent in all the 9 southern positive lines under both the promoters, indicating the expression of transgene in all the transgenic lines tested (Fig. 3B).

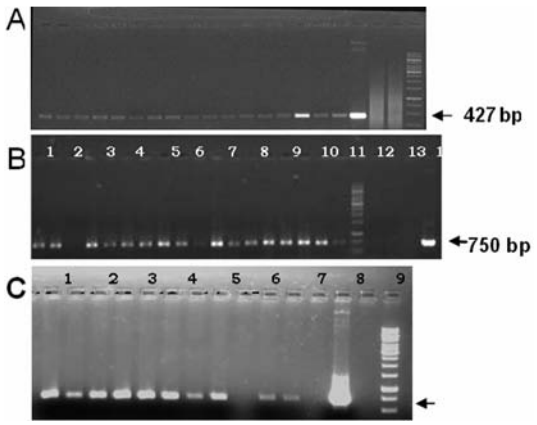

Fig. 2—(A) PCR amplification of T_3 transgenic lines with gene specific primer (*DcCs*). [Lanes 1-12, $AtPT_2$::*DcCs* construct; 13-17, *CaMv*::*DcCs*; 18, Plasmid; 19, Wild type; 20, Blank; and 21, marker]. (B) PCR amplification of T_3 transgenic lines with nptII primer. [Lanes 1-12, *AtPT2*::*DcCs*; 13-18, *CaMv*::*DcCs*; 19, Marker; 20, Blank; 21 & 22, Wild type; 23, Plasmid]. (C) PCR of T_3 transgenic plants with primers specific to Promoter region. [Lanes 1-8, 10-12, Transgenic lines; 9, Wild type; 13, Plasmid; 14, Blank; and 15, marker]

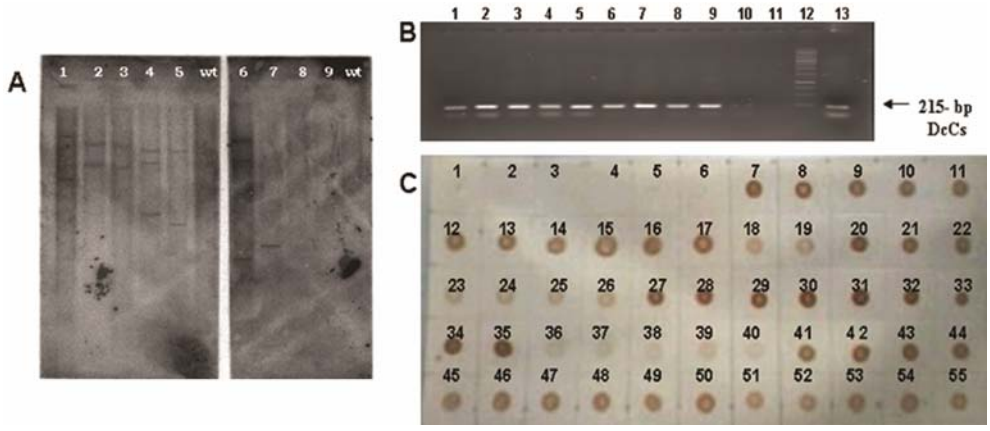

Fig. 3—(A) Southern analyses of the transgenic lines showing copy number. The probe used was *nptII*. Lanes 1-5 are transgenic plants of $AtPT_2$ construct (1= 1-3-1, 2=2-2-4, 3=4-2-3, 4=7-5-3, 5=8-3-2) and 6-8 are transgenic plants of *CaMV*::*DcCs* construct (6= 1-2-4, 7= 3-1-3, 8= 6-2-3, 9= 10-2-1); (B) Gene expression analysis of *DcCs* through RT-PCR in transgenic lines over expressing *DcCs* under Constitutive and root specific promoters.Lanes 1-5: transgenic plants of $AtPT_2$ construct and 6-9: transgenic plants of *CaMV*::*DcCs* onstruct. 10: Wild type, 11: Blank, 12: marker (1Kb), 13: Palsmid; and (C) Dot blot analysis of transgenic pigeonpea plants over expressing citrate synthase gene. 1, 2, 3 = BSA (negative control), 4, 5, 6 = with out antigen (blank), 7-22 =transgenic lines ($AtPT_2$::*DcCs* construct) &27-35(*CaMV*::*DcCs*) construct, grown under P deficient conditions. 23-26 = non transgenic lines grown with out phosphorus, 36-40 = non transgenic lines grown with phosphorus, 41-49 = transgenic lines ($AtPT_2$::*DcCs* construct) & 50-55 transgenic lines (*CaMV*::*DcCs*) construct) grown with phosphorus

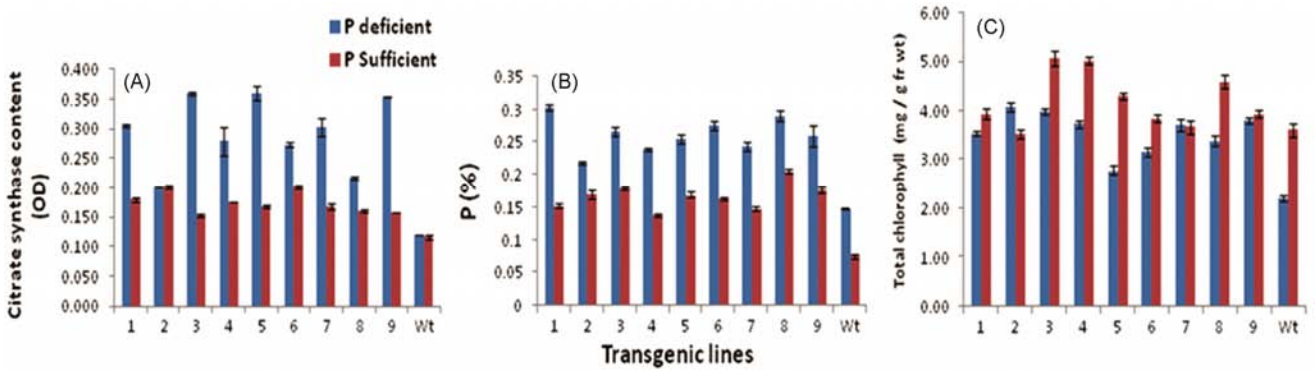

Fig. 4—(A) Citrate synthase content of transgenic plants under P sufficient and deficient conditions determined through ELISA; (B) Tissue P concentrations in the transgenic and non transgenic plants as influenced by phosphorus; and (C) Total chlorophyll content in the transgenic plants compared with the wild type. The data given here is from independent representative experiments.

Dot blot analysis

To further support our data, a dot blot analysis was performed with polyclonal antibodies developed at our lab for citrate synthase. Fig. 3C indicates significant variation among the different transgenic lines. A higher degree of expression was observed in the selected transgenic lines, both under constitutive and root specific promoters when compared to wild type plants, under P deficient conditions. The expression levels were higher in lines 27-35 belonging to *CaMV*::*DcCs* construct.

Physiological analysis

Expression analysis of citrate synthase

The citrate synthase enzyme was quantified through ELISA technique. We observed that the transgenic plants over-expressing citrate synthase gene had more citrate synthase under P deficient conditions than the wild type plants. There was a 3 fold increase in this enzyme in some of the transgenic lines over the wild type (Fig. 4A). Lines 4-2-3 & 8-3-2 of *AtPT*$_2$::*DcCs* construct and line 10-2-1 of *CaMV*::*DcCs* had the highest content of citrate synthase

Tissue P concentration

The tissue P content of all the transgenic lines was higher both under P sufficient as well as deficient conditions than the wild type. However, it was highest in the transgenic lines under P deficient conditions (Fig. 4B). Line 1-3-1 belonging to *AtPT2*::*DcCs* construct had the highest P concentration followed by lines 6-2-3 and 1-2-4 belonging to *CaMV*::*DcCs* construct.

Chlorophyll content

Both in the transgenic lines and the wild type plants, the total chlorophyll content was higher under P sufficient conditions when compared to the deficient conditions. However, all the transgenic lines had high chlorophyll content than the wild type both under P sufficient and deficient conditions (Fig. 4C).

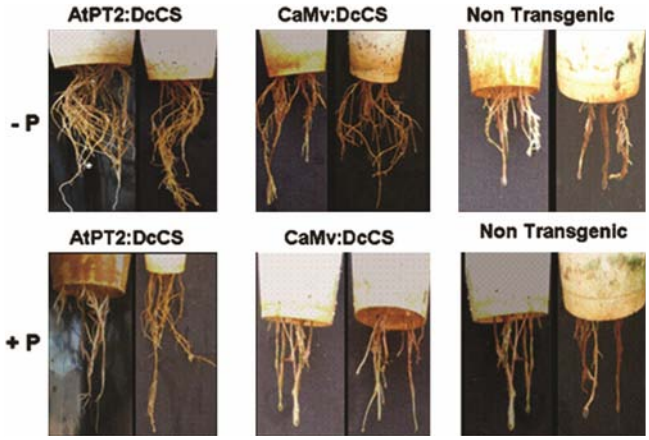

Fig. 5—Variation among the transgenic and non transgenic plants in root growth under P sufficient and deficient conditions.

Root growth

Considering the fact that deficiency of P modifies the root architecture, influence of P deficiency and the over-expression of citrate synthase gene on the root architecture were examined. We found that both deficiency of phosphorus and over-expression of citrate synthase, individually and together, resulted in increased number of lateral roots and root hairs. Only visual differences were observed (Fig. 5). Data on root length or volume or dry wt. could not be recorded as the transgenic plants could not be sacrificed as mentioned earlier.

Discussion

Phosphorus (P) is one of the most limiting nutrients for plant growth in many natural and agricultural ecosystems. P acquisition by plants is usually a

constraint for plant productivity because, the inorganic form of phosphate (Pi) that is taken up by roots, is unevenly distributed and relatively immobile in soil due to its affinity for cations and its conversion to organic forms[21]. In plants, a general strategy to cope up with low Pi availability has been described, which involves 3 fundamental mechanisms: (i) release and uptake of Pi from external organic and inorganic sources[22]; (ii) optimization of Pi use by a wide range of metabolic alterations and the mobilization of internal Pi[3,23]; and (iii) an increase in the exploratory capacity of the root and the absorptive surface area by altering the root system architecture[24,25]. Increased synthesis and release of organic acids like citrate or malate is a part of the first mechanism adopted by plants to acquire more P under deficient conditions. Enhanced synthesis of citrate can be achieved by enhancing the content and activity of enzyme citrate synthase[26]. It is well established that under P deficient conditions, few key enzymes of citric acid cycle either get upregulated or downregulated[12]. Isocitrate dehydrogenase and aconitase are among the downstream enzymes downregulated, while citrate synthase and PEP carboxylase are upregulated[12]. Upregulated enzymes increase the citrate synthesis while downregulated ones increase its accumulation in the cytoplasm which is thrown out into the apoplasm, and then to the rhizosphere where it solubilizes the inorganically bound P.

Transgenic approaches to enhance the citrate synthesis by over-expressing genes encoding citrate synthase has been attempted by several workers[12,26]. The efficiency of such transgenic plants to acquire more phosphorus under phosphorus deficient conditions by solubilizing inorganically bound phosphorus has also been reported[7,26]

Considering these facts, here we attempted to develop transgenic pigeonpea plants over-expressing citrate synthase gene. Pigeonpea being an important pulse crop of the arid and semiarid tropics and an excellent source of vegetarian protein was the choice of the study. Since pigeonpea is recalcitrant to tissue culture, we adopted tissue culture independent technique called the *in-planta* transformation. This technique was first standardized in Arabidopsis[27] and methodology was extended to other crops that are not amenable to tissue culture, such as rice[28], Soybean[29] and peanut[13].

The major objective of this study was to develop transgenic Pigeonpea plants over-expressing citrate synthase gene cloned from *Dacus carota* (*DcCs*) under 2 different promoters, a root specific promoter ($AtPT_2$) and a constitutive (*CaMV-35S*) promoter. Molecular analysis of T_1, T_2 and T_3 generations indicated integration of the transgene.

The transgenic nature of plants was confirmed by physiological and molecular analysis of the transgenic plants. Gene expression was confirmed through RT-PCR and dot blot analysis, supported by biochemical analysis through quantification of citrate synthase, chlorophyll and tissue P content. We noticed higher content of citrate synthase in the transgenic lines when compared to wild type plants under phosphorus limited conditions. Similar study by Koyama *et al*.[26] also reported 3-4 fold increase in citrate synthase enzyme and corresponding increase in fresh weights of the plants. Further investigations revealed that transgenic plants have high tissue P and chlorophyll contents. Over-expression of citrate synthase gene increased fresh weight[26], root length, citrate synthase activity[26], contents of chlorophyll[30], and citrate acid in transgenic Arabidopsis[26].

A large root system capable of exploring greater soil volume has been recognized as one of the important adaptations of plants to ensure sufficient uptake of P[31]. The root architecture of plants can undergo several changes in response to P deficiency. Increases in lateral root growth and secondary root branching at the expense of primary root elongation was observed in beans[31] and Arabidopsis[32]. In this study, it was observed that the transgenic plants produced more lateral roots than the wild under –P conditions (Fig. 5). It is also suggested that deficiency of P upregulates auxin synthesis[3,33]. Hormones are reported to play key role in mediating P starvation effects on root system architecture[34]. The mediation of phosphorus starvation is suggested to be by an over-accumulation of auxin in the apex of primary root and in young lateral roots. Further, it could also be a change in sensitivity to auxins in the lateral primordia. A decrease in the auxin concentration in the lateral primordia initiation zone of the primary root and old laterals is also suggested[35].

Conclusion

We conclude from this study that pigeonpea transgenic plants can be successfully developed following a non tissue culture method and that the transgenic Pigeonpea plants over-expressing citrate synthase gene are capable of acquiring more phosphorus under phosphorus deficient conditions by virtue of their capability to synthesize more citrate

synthase and produce more lateral roots. The expression levels were higher in the transgenic plants where construct with root specific promoter was used when compared to the constitutive promoter. Though we got both single and multiple copies of the gene, the RT-PCR analysis indicated better expression of the transgene and that there was no silencing of the gene due the presence of multiple copies. However, a single copy of the gene as noticed in one of the lines is always preferable. The transgenic lines developed in this study can be grown successfully under phosphorus deficient conditions without any added phosphatic fertilizers. Further investigations to test the capability of these transgenic plants to access P bound either to Ca, Mg, Fe or Al would be rewarding.

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References

1 Raghothama KG, Phosphate acquisition. *Ann Rev Plant Physiol Plant Mol Biol*, 50 (1999) 665.
2 Fitter AH & Hay RKM, *Environmental Physiology of Plants*, (Academic Press, London), 2002.
3 Vance CP, Uhde-Stone C & Allan DL, Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytol*, 157 (2003) 423.
4 Shane MW & Lambers H, Cluster roots: a curiosity in context. *Plant Soil*, 274 (2005) 101.
5 López-Bucio J, de la Vega OM, Guevara-García A & Herrera-Estrella L, Enhanced phosphorus uptake in transgenic tobacco plants that overproduce Citrate, *Nat Biotechnol*, 18 (2000) 450.
6 Shen J, Li h, Neumann G & Zhang F, Nutrient uptake, cluster root formation and exudation of protons and citrate in *Lupinus albus* as affected by localized supply of phosphorus in a split-root system. *Plant Sci*, 168 (2003) 837.
7 Ryan PR, Delhaize E & Jones DL, Function and mechanism of organic anion exudation from plant roots. *Ann Rev Plant Physiol Plant Mol Biol*, 52 (2001) 527.
8 Singh B & Pandey R, Root exudation by P-starved Maize genotypes and its relationship with P uptake. *Indian J Plant Physiol*, 7 (2002) 187.
9 Tran HT, Hurley BA, Plaxtona & WC, Feeding hungry plants: the role of purple acid phosphatases in phosphate nutrition. *Plant Sci*, 10 (2010) 1016.
10 Yan-Su Li, Yan Gao, Qiu-Ying Tian, Feng-Ling Shi, Ling-Hao Li & Wen-Hao Zhang, Stimulation of root acid phosphatase by phosphorus deficiency is regulated by ethylene in Medicago falcate. *Environ Exp Bot*, 71(2011) 114.
11 Neumann G, Agnès Massonneau, Nicolas Langlade, Barbara inkelaker, Christine Hengeler, Volker Römheld & Enrico Martinoia, Physiological Aspects of Cluster Root Function and Development in Phosphorus-deficient White Lupin (*Lupinus albus* L.). *Annals Bot*, 85 (2000) 909.
12 Tomonori Kihara, Tatsumi Wada, Yuji Suzuki, Tetsuo Hara & Hiroyuki Koyama, Alteration of Citrate Metabolism in Cluster Roots of White Lupin. *Plant Cell Physiol*, 44 (2003) 901.
13 Rohini VK & Rao KS, Transformation of peanut (*Arachis hypogaea* L.) a non-tissue culture based approach for generating transgenic plants. *Plant Sci*, 150 (2000) 41.
14 Sambrook J & Russell DW, Extraction, purification, and analysis of mRNA from eukaryotic cells. In: *Molecular cloning: A laboratory manual*. Vol 2, 2nd ed. (Cold Spring Harbour Laboratory Press, New York), 1989.
15 Dellaporta SL, Wood J & Hicks JB, In: *Molecular Biology of Plants: A Laboratory Course Manual*. (Cold Spring Harbor, New York), 1984, 36.
16 Southern EM, Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol*, 98 (1975) 503.
17 Destefano JJ, Buiser RG, Mallaber LM, Myers TW, Bambara RA & Fay PJ, Polymerization and RNase H activities of the reverse transcriptases from avian myeloblastosis, human immunodeficiency, and Moloney murine leukemia viruses are functionally uncoupled. *J Biol Chem*, 266 (1991) 7423.
18 Fonseca ME, Marcellino LH & Gander E, A rapid and sensitive dot-blot hybridization assay for the detection of citrus exocortis viroid in Citrus medica with digoxigenin-labelled RNA probes. *J Virol Methods*, 57 (1996) 203.
19 Hobbs HA, Reddy DVR, Rajeshwari R & Reddy AS, Use of Direct Antigen Coating and protein A coating ELISA procedure for detection of three peanut viruses. *Plant Dis*, 71 (1987) 747.749
20 Hiscox JD & Israelstam GF, A method for the extraction of chlorophyll from leaf tissue without maceration. *Can J Bot*, 57 (1979) 1332.
21 Holford ICR, Soil phosphorus: Its measurement and its uptake by plants. *Aust J Soil Res*, 35 (1997) 227.
22 Karthikeyan AS, Varadarajan DK, Mukatira UT, D'Urzo MP, Damsz B & Raghothama KG, Regulated expression of Arabidopsis phosphate transporters. *Plant Physiol*, 130 (2002) 221.
23 Cruz-Ramírez A, Oropeza-Aburto A, Razo-Hernández F, Ramírez-Chávez E & Herrera-Estrella L, Phospholipase DZ2 plays an important role in extraplastidic galactolipid biosynthesis and phosphate recycling in Arabidopsis roots. *Proc Natl Acad Sci USA*, 103 (2006) 6765.
24 López-Bucio J, Herna´ndez-Abreu E, Sánchez-Calderón L, Nieto-Jacobo MF, Simpson J & Herrera-Estrella L, Phosphate availability alters architecture and causes changes in hormone sensitivity in the Arabidopsis root system. *Plant Physiol*, 129 (2002) 244.
25 Sánchez-Caldero´n L, López-Bucio J, Chaco´n-López A, Cruz-Ramírez A, Nieto-Jacobo F, Dubrovsky JG & Herrera-Estrella L, Phosphate starvation induces a determinate developmental program in the roots of *Arabidopsis thaliana*. *Plant Cell Physiol*, 46 (2005) 174.
26 Hiroyuki Koyama, Ayako Kawamura, Tomonori Kihara, Tetsuo Hara, Eiji Takita & Daisuke Shibata, Overexpression of Mitochondrial Citrate Synthase in *Arabidopsis thaliana* Improved Growth on a Phosphorus-Limited Soil. *Plant Cell Physiol*, 41 (2000) 1030.

27 Feldmann KA & Marks MD, *Agrobacterium* mediated transformation of germinating seeds of *Arabidopsis thaliana*: A non-tissue culture approach. *Mol Gen Genet*, 208 (1987) 1.

28 Supartana P, Shimizu T, Shioiri H, Nogawa M, Nozue M & Kojima M, Development of simple and efficient *in planta* transformation method for rice (*Oryza sativa* L.) using *Agrobacterium tumefaciens*. *J Biosci Bioengg*, 100 (2005) 391.

29 Chee PP, Fober AK & Slightom LJ, Transformation of soybean [Glycine max (L.) Merrill] by infecting germinating seeds with *Agrobacterium tumefaciens. Plan Physiol*, 91 (1989) 1212.

30 Jiang HM, Yang JC & Zhang JF, Effects of external phosphorus on the cell ultrastructure and the chlorophyll content of maize under cadmium and zinc stress. *Environ Pollut*, 147 (2007) 750.

31 Sattelmacher B, Horst WJ & Becker HC, Factors that contribute to genetic variation for nutrient efficiency of crop plants. *J Plant Nutr Soil Sci*, 57 (1994) 215.

32 Horst WJ, Kamh M, Jibrin JM & Chude VO,. Agronomic measures for increasing P availability to crops. *Plant Soil*, 237 (2001) 21.

33 Ticconi CA, Delatorre CA, Lahner B, Salt DE & Abel S, Arabidopsis pdr2 reveals a phosphate-sensitive checkpoint in root development. *Plant J*, 37 (2004) 801.

34 Nacry P, Genevie`ve Canivenc, Bertrand Muller, Abdelkrim Azmi, Harry Van Onckelen, Michel Rossignol & Patrick Doumas, A Role for Auxin redistribution in the responses of the root system architecture to phosphate starvation in Arabidopsis. *Plant Physiol*, 138 (2005) 2061.

35 Yamagishi M, Hou K, Osaki M, Miller SS & Vance CP, Real-time RTPCR profiling of transcription factors including 34 MYBs and signaling components in White lupin reveals their P status dependent and organ-specific expression. *Plant Soil*, 342 (2011) 481.